Instituto Juan March
de Estudios e Investigaciones

125 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Left-Right Asymmetry

Organized by

C. J. Tabin and J. C. Izpisúa Belmonte

M. Averof
M. Blum
M. Brueckner
J. Cooke
J. Goodship
M. Halpern
H. Hamada
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M. R. Kuehn
M. Levin
C. McManus
M. Mercola
A. H. Monsoro-Burq
M. Ros
A. F. Schier
T. Strachan
C. J. Tabin
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Introduction
J. C. Izpisúa Belmonte

A fundamental aspect of vertebrate embryogenesis are the events responsible for patterning the embryo as it develops from a single-celled fertilized egg into a complex multicellular organism. Understanding the mechanisms responsible for distinguishing the left and right sides of the embryo relative to its anterior-posterior and dorsal-ventral axes has been a main focus of research recently. Although vertebrates are essentially bilaterally symmetrical with respect to their external features, very dramatic internal asymmetries develop about their midline axis during embryogenesis. For instance, the heart, stomach and spleen are invariably on the left, while nearly all of the liver is on the right. The positioning of the internal organs with respect to the midline is highly conserved between species and is referred to as *situs solitus*. Alterations in this classic pattern may occur as a complete mirror image reversal of all of the organs (*situs inversus*), reversal of individual organs along the left-right axis (heterotaxia), or changes in normal symmetry or aberrant bilateral symmetry of a particular organ (isomerism). Except for complete *situs inversus*, the physiological consequence of laterality defects in internal organ positioning is usually severe. The series of embryonic developmental events that distinguish the left and right sides of the embryo along its anterior-posterior and dorsal-ventral axes include the direction of axial rotation, morphogenesis of individual organs, and organ placement.

The findings derived from research aimed at characterizing the molecules responsible for patterning the left-right axis of the embryo formed the basis of a workshop held at the Juan March Foundation in Madrid between June 3-6, 2001.

Current and future challenges in understanding left right asymmetry were discussed. They included the question of how the early bilateral symmetry of the embryo is broken, and how this initial asymmetry isconverted into much broader domains of site specific expression that subsequently coordinate the asymmetric development of the various organ primordia. Profound insights emerged regarding the molecular mechanisms by which these broad domain of asymmetric gene expression are confined and regulated to their original side and how, as development proceeds, they subsequently translate into asymmetric organ development. The commonalities, but perhaps more importantly, the differences among animal in the mechanisms that establish left right asymmetry were thoroughly discussed.

One of the most intriguing questions discussed in the workshop concerned the relationship between the left right patterning of the visceral organs and the phenomenon of brain lateralization. Last, but no least, the etiology of human laterality disorders highlighted the ongoing synergism between humans and the various vertebrate model organisms and pointed directions to allow rapid advances in our understanding of the global mechanisms underlying left right pattern formation in vertebrates.

J. C. Izpisúa Belmonte

Session 1: Breaking symmetry
Chair: Cliff J. Tabin

Current and future challenges in understanding left right asymmetry

Kyle Vogan, Tina Bruekner, Joe Yost, Nikki Davis, Richard Pearse, Richard Harvey and Cliff J. Tabin

Only in the last decade have researchers been able to link specific gene functions to particular processes during the development of Left-Right asymmetry along the medial-lateral axis. The existence of mouse, zebrafish and human mutants with distinct la6terality defects had suggested that Left-Right specification was under genetic control. This expectation was confirmed by the identification of several genes showing side-specific patterns of expression in the early embryo whose activities could affect morphological assymetry (reviewed in Capdevilla et al., Cell 101, 9-21, 2000). The challenges we currently face are to place these genetic pathways into teh context of several important events in the process of Left-Righht determination, including identification of the initial breaking of symmetry and how that event links to downstream genetic cascades, a deeper understanding of the unilateral signaling cascades that propogate this information, and uncovering the cellular responses which convert these signals into asymmetric morphologenesis. Approaches to all three of these issues will be discussed, including examination of the phylogenetic conservation of cilia at the node which have been hypothesized to be responsible for initiating Left-Right asymmetry in the mouse, the identification of two branches of Hedgehog signaling in the left-side cascade in the chick, and a screen to identify asymmetric cellular responses in the cardiac primordium involved in asymemtric morphogenesis of the heart tube.

The role of ion channels and pumps in embryonic left-right asymmetry

Michael Levin

The proper establishment of left-right (LR) asymmetry depends, in part, on a cascade of asymmetrically-expressed genes which regulate each other's expression on either side of the midline to ultimately control the *situs* of the visceral organs (Levin, 1998). This asymmetric gene expression requires fields of cells to know on which side of the embryonic midline they are located. In contrast, the initial step of LR asymmetry is likely to be due to the chirality of a subcellular component (Brown and Wolpert, 1990), which when tethered with respect to the AP and DV axes, provides the orientation of the LR axis. Thus, we need to understand the mechanisms which convert information on which direction is L and which R to information on a cell's location relative to the embryo's midline.

To begin to understand mechanisms which allow cells to ascertain their position within the context of the whole embryo, we investigated the role of gap junctions in embryonic LR asymmetry. We showed (Levin and Mercola, 1998; Levin and Mercola, 1999) that in both chick and frog embryos, a system of gap junctional communication (GJC) functions upstream of asymmetric gene expression in LR patterning. Large-scale (embryo-wide) patterns of GJC exist at early stages, as detected directly by junctional transfer of fluorescent dyes and expression of *Connexin* family genes; these GJC patterns are circumferential and terminate in a zone of junctional isolation at the embryonic midline. The zone of isolation is the primitive streak in chicks, and the ventral midline in *Xenopus*. Using surgical manipulation, chemical agents which target gap junctions, and specific misexpression of dominant-negative and constitutively-active *Connexin* constructs, we demonstrated that the integrity of the open junctional path, as well as the zone of isolation, is crucial for correct LR asymmetry of gene expression and organ *situs*. This system functions early in development (blastula to early gastrula stages) and is a general mechanism for orienting the LR axis within the embryo (it is not specific to the morphogenesis of any of the asymmetric organs *per se*).

Our current model is that small molecule morphogens traverse the circumferential junctional paths in a chiral (unidirectional) way. By preferentially flowing in one direction, they accumulate in a gradient to one side of the zone of isolation and then induce asymmetric gene expression on either side of the midline. The morphogens are as yet unidentified, but my lab is pursuing a likely candidate. Here, I present a summary of our work addressing another crucial question: what is responsible for the chiral (unidirectional) flow? One model involves unidirectional gap junctions, which permit the passage of permeant molecules in only one direction (Robinson et al., 1993; Xin and Bloomfield, 1997). We have evidence of these in embryos, but they cannot constitute the full explanation for chiral flow because thermodynamics forbids the generation of gradients with trap-doors (Maxwell's demon). Since gap junctions consume no energy, some energetic mechanism has to be involved in the chiral flow. We hypothesized that perhaps voltage potential differences on either side of the zone of isolation electrophorese charged determinants through the junctional path.

Standing potential voltage differences have been observed in many embryonic systems (Borgens et al., 1989; Hotary and Robinson, 1992; Hotary and Robinson, 1994; Robinson and Messerli, 1996), and are thought to direct a number of aspects of morphogenesis (such as galvanotactic guidance of cell migration). These fields are generated by the function of ion

pumps (which produce voltage gradients at the expense of ATP) and shaped by ion channels. In order to test our electrophoresis model, we took advantage of the plethora of specific pharmacological agents which have been developed by the neurobiology community to target ion channels and pumps. Our model predicts that inhibition of specific ion channel or pump activity will disrupt laterality. By performing a pharmacological screen, we found that inhibition of H^+ and K^+ transport, but not Cl^-, Na^+, or Ca^{++} transport, specifically induces a high incidence of heterotaxia in *Xenopus* embryos in the absence of other defects. By using increasingly specific reagents, we narrowed the candidate proteins further to the K^+ channel KvLQT-1 and the H^+ pump (V-ATPase).

Our model also predicts that these ion flows are important during early steps of asymmetry, roughly corresponding to the stages we found for the GJC system. By applying the drug reagents at different developmental times, we found that heterotaxia results when the appropriate pump or channel is inactivated during blastula stages in frog, and the early primitive streak stage in chick. Consistent with the early time window, we found that asymmetric expression of genes such as *Shh* and *xNR-1* is destabilized when V-ATPases are inhibited during early stages.

Our model makes a very specific prediction about the expression of the ion pumps and channels which participate in LR patterning. To serve as a battery driving electrophoresis through the open circuit formed by the GJC path, ion pumps must be expressed at the zone of isolation, during the stages defined by the pharmacology; the opposite polarity must be accomplished by some asymmetry in their function (which could exist at the mRNA or protein regulatory levels). Guided by the pharmacology, we examined the expression pattern of a number of candidate genes. As predicted, we found expression of K^- channel and H^+ pump genes in the primitive streak of st. 2 chick embryos, and in the ventral midline of *Xenopus embryos*. Interestingly, we found a LR asymmetry in the expression of a V-ATPase subunit at the ventral midline of the 4-cell stage in frog embryos; this is the earliest asymmetry reported in the literature. We are currently investigating cytoplasmic transport events which might be responsible for this asymmetry in mRNA localization.

Our model makes a further prediction: that ion flows and potential voltage differences exist at the zone of isolation, and further, that these will be asymmetric across the LR midline. In order to directly observe these phenomena, we collaborated with a number of investigators. Using sophisticated fluorescent imaging and electrophysiological techniques appropriate to chick and frog systems, we found that $V_{membrane}$, H^+ efflux, and pH all exhibit consistent LR asymmetries at the zone of isolation. These asymmetries are abolished by pharmacological reagents which we previously showed induce heterotaxia.

We are currently using a variety of constructs encoding wild-type, constitutively-active, and dominant-negative channel and pump constructs to refine our model. We are also continuing the electrophysiology and imaging to gain a better understanding of the role of endogenous ion currents in embryonic morphogenesis. Finally, we are investigating the possible involvement of candidate small molecule morphogens which traverse gap junctions in LR patterning, and the cytoplasmic transport events which are upstream of ion pump asymmetry. It is possible that motor proteins function in LR asymmetry through a role in cytoplasmic transport of ion channels and pumps at early stages of development.

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Mutation of the N-terminus of left-right dynein (lrd) results in mice with non-random reversal of left-right asymmetry

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Movement of monocilia found on ventral node cells of e7.5 mouse embryos is associated with directional flow of perinodal fluid (nodal flow) and generation of handed left-right (LR) asymmetry. Previously, we identified an axonemal dynein motor, lrd, required for node cilia movement: when the 1st ATPase domain of lrd is mutated, node cilia are paralyzed and LR asymmetry becomes random. We have now generated a targeted mutation in the N-terminal region of lrd, called lrdGFPΔneo. This mutation fuses Green Flourescent Protein (GFP) with lrd immediately following the lrd start codon. RT-PCR analysis of mRNA from lrdGFPΔneo mice shows lrd mRNA from the start of lrd through the GFP coding region that is spliced into the neo-r gene, and lrd mRNA extending from the 2nd exon to the 3' end of lrd. Western blot analysis of e7.5 lrdGFPΔneo embryos with an anti-lrd antibody shows several truncated lrd proteins. Unlike mice with mutations in the ATPase domain of lrd, lrdGFPΔneo mice have non-random reversal of LR asymmetry. 38 of 54 lrdGFPΔneo -/- mice were situs inversus, differing significantly from the expected 50% with a p-value of .0028. Average litter size of lrdGFPΔneo mice is 5, indicating embryonic lethality. The *inv* -/- mutation is the only other known mouse with > 50%situs inversus. *Inv* -/- mice have normal direction of node ciliary movement, but slow nodal flow. These observations raise the possibility that lrdGFPΔneo mice may also have slow nodal flow. This could be due to either a hypofunctional ciliary dynein motor, or to abnormal binding of regulatory dynein light and medium chains to the mutant N-terminal region of lrd.

Nodal flow and left-right asymmetry: Lessons from gene targeting of KIF 3. Molecular motor and analysis of iv and inv mice

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KIF 3 is a member of the kinesin superfamily motors composed of KIF 3A/3B heterodimer and an associated protein, KAP 3 and is expressed ubiquitously. To elucidate the function of KIF 3 we performed knockout of kif 3a and kif 3b. kif 3a-/- or kif 3b-/- mice were embryonal lethal and displayed severe developmental abnormalities. Prominently, the left-right asymmetry was randomized. The L-R asymmetry is already detectable at the somitogenesis stage by asymmetric expression of several genes, such as lefty 2, lefty 1, and nodal in the left side. In these mutants the most upstream gene, lefty 2 expression was either bilateral or undetectable. Thus, KIF 3 relates to phenomena upstream to this gene cascade. Then we examined the node, important for L-R determination. Although the nodal cells of wild type have monocilia, cilia were either completely absent or very short in mutant embryos. In addition immunocytochemistry showed KIF 3 are localized in monocilia of the node cells of wild type. The data suggest that KIF 3 conveys protein complexes for the ciliogenesis along the microtubules in the cilia in node cells. Furthermore, surprisingly enough, the monocilia, previously thought to be immotile, were vigorously rotating in wild type and these movement generated unidirectional leftward flow of extraembryonic fluid named as nodal flow. However, in mutants there was no nodal flow. These data suggested that KIF 3 is essential for the L-R determination through intraciliary transportation of protein complexes for ciliogenesis of motile primary cilia that could produce a gradient of putative morphogen along the L-R axis in the node. We examined also the nodal flow of well-characterized mouse mutants, iv and inv and found that their laterality defects are always accompanied by an abnormality in nodal flow. In a randomized laterality mutant, iv, the nodal cilia were immotile and the nodal flow was absent. In a situs inversus mutant, inv, the nodal cilia was motile but could only produce very weak leftward nodal flow. These results consistently support our hypothesis that the nodal flow produces the gradient of putative secreted morphogen and triggers the first L-R determination event.

Session 2: Early establishment of asymmetry
Chair: H. Joseph Yost

Approaching the function of inversin: from gene structure and transcript studies to evolutionary analyses and screens for interacting proteins

Tom Strachan

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The recessive *inv* mutation was generated by transgene insertion into mouse chromosome 4 and is characterised by consistent reversal of the left-right axis: virtually all (95%) homozygotes have global situs inversus including reversed heart looping, and 5% have evidence of isomerism and splenic anomalies [1]. The kidneys are large and pale with marked dilatation of the tubules and collecting ducts. There is expansion and disorganisation of the endocrine cells in the islets of Langerhans in the pancreas and apparent decrease in the number of exocrine acinar cells. The mice have extrahepatic biliary obstruction [2], develop jaundice and die approximately a week after birth. In collaboration with Dr. Paul Overbeek and colleagues we used a positional cloning approach to identify a novel gene, *Inversin*, as the locus for the causative mutation. *Inversin* has 16 exons and we showed that the transgene insertion results in a 47 kb deletion that removes exons 3-12 [3]. At the same time an independent study by Yokoyama *et al.*, reported cloning of this gene, showing that it was sufficient to account for the *inv* phenotype [4]. The inversin protein is 1062 amino acids long and has 16 tandem ankyrin repeats spanning amino acids 13-557; otherwise the sequence has revealed few clues to its function. *In situ* hybridisation and RT-PCR studies have shown that although the inversin gene is transcribed at early stages in mouse development, expression appears to be virtually ubiquitous and at low levels. Northern hybridization analyses revealed a single 5.7 kb transcript in a variety of adult tissues while RT-PCR analysis suggested the possibility of 3 isoforms.

We carried out evolutionary screens to identify highly conserved regions, and yeast two hybrid screens to identify interacting proteins. We were unable to identify orthologues in *C.elegans* or in *D. melanogaster* but in collaboration with Drs. Cliff Tabin and Jo Yost inversin sequences were obtained for chick, Xenopus, and zebrafish. The available data shows that the N terminal region including the ankyrin repeat region is highly conserved, as is a segment of about 50 amino acids commencing at residue 558, immediately downstream of the ankyrin repeat region. Within the latter segment we noted a calmodulin-binding IQ motif and inspection of the rest of the sequence revealed a second highly conserved motif of this type located in the otherwise poorly conserved C terminal region. We designed a series of yeast two hybrid screens. Initially we used an inversin bait to screen a mouse 7 day embryo cDNA library and found that 36% of the positive clones encoded calmodulin. We made a series of inversin binding domain baits and tested these with a calmodulin activation domain construct. The latter construct failed to bind to an inversin construct comprising the ankyrin repeat region alone but did bind a reciprocal inversin construct lacking the ankyrin repeat region but retaining the two IQ motifs. Validation of the calmodulin-inversin interaction was obtained by co-immunoprecipitation experiments.

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Approaching the function of inversin: protein expression studies

Judith Goodship

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The sequence of the recently isolated *Inversin* gene has revealed few clues to its function. We have studied intracellular localisation in NIH3T3, IMCD3 renal collecting duct cells and MDCK renal epithelial cells by GFP tagging and using a polyclonal inversin-specific antibody. In NIH3T3 cells we observed a dynamic expression pattern. In non-dividing cells three patterns were observed: i) a lattice-like network around the nucleus; ii) a suggestion of more condensed perinuclear staining; and iii) centrosomal localisation. In dividing cells we observed expression at the spindle poles. To investigate the expression in dividing cells further we injected an RNA inversin-GFP construct into sea urchin eggs. This confirmed the expression along mitotic spindles and at spindle poles. A surprising and consistent finding as the blastula developed was that GFP expression was strong in the aboral region and absent from the oral region. The mechanism by which this asymmetry of expression arises is not clear. MDCK cells develop primary cilia as they become confluent in culture. Intensity of inversin signal increases dramatically as the cells become confluent. Expression is then observed at the base of the cilium and along the cilium. This pattern is similar to the expression profile of polaris in MDCK cells [1]. *Tg737*, the gene encoding polaris, was cloned in the insertional transgenic *oak ridge polycystic kidney* mouse and inactivation of this gene results in randomisation of the left-right axis and absent nodal cilia [2]. Thus inversin localizes in cilia but is not required for generation of cilia. We postulate that the primary cilia in the kidney are flow sensors and that calcium signaling is the means of signal transduction effecting changes in cell polarity. The observed interaction between inversin and calmodulin is consistent with involvement of inversin in such a pathway. How these observations relate to events at the node is unclear and the consistent reversal of the left-right axis in the *inv* mouse remains an enigma.

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BMP4 plays a key role in left-right patterning in chick embryos by maintaining sonic hedgehog asymmetry

Anne-Hélène Monsoro-Burq

Early left-right polarisation events can be divided into three steps in the chick embryo: an inductive period when the environment imposes its initial polarity on Hensen's node, a determination step when the L/R axis is fixed in the node itself and a propagation period when the node transmits its polarity information to the lateral mesodermal tissues. The earliest signs of asymmetry known in chicks are the polarised expressions of several genes on each side of Hensen's node: *Shh* on the left (from stage 4+), *Activin βB* (stage 3), *Activin-R IIa* (stage 4) and *Fgf8* (stage 6) on the right (Levin et al., 1995; 1997; Boettger et al., 1999). From stages 4 to 5+, *Shh* asymmetry is still labile whereas at stage 6 it is determined, indicating that the L/R axis is fixed by this time (Pagàn Westphal and Tabin, 1998).

Among the questions remaining open, we were particularly interested in the early molecular mechanisms involved in the determination of the L/R axis, and in the coordination between the left and the right side.

We initially observed the asymmetrical expression of several members of the BMP signalling cascade on the right side of Hensen's node (Monsoro-Burq and Le Douarin, 2000): among them, *Bmp4* gene is expressed on the right side of the node from stage 5- to stage 7. We first analysed the regulation of *Bmp4* gene transcription *in vivo* by implanting sources of recombinant factors nearby the node. We show that Activin A is able to upregulate *Bmp4* expression ectopically on the left, whereas SHH blocks *Bmp4* gene transcription on the right.

Second, we tested the role of BMP4 either by implanting sources of recombinant BMPs ectopically or by blocking endogenous BMP signalling by the specific antagonist Noggin. We demonstrate that BMP4 signalling is both necessary and sufficient to maintain the asymmetry initiated in *Shh* gene expression. In the absence of BMP signalling, *Shh* reappears on both sides of the node, with a symmetrical pattern. Similarly, we show that BMP4 is necessary and sufficient to activate *Fgf8* gene on the right side of the node.

The analysis of embryos operated at stage 4+ later during development shows the absence of *Nodal* on the left paraxial mesoderm, the ectopic activation of *Snail* (Isaac et al., 1997) on the right and finally randomisation of the heart situs.

We thus present a model of the molecular mechanisms controlling determination of the L/R polarity in the node: a negative regulatory loop implicating SHH and BMP4 arises at stage 5- and stabilizes the still labile asymmetries imposed by the environment. Moreover proper BMP4 levels coordinate the proper distribution of *Shh* and *Fgf8* genes on each sides of the node, establishing the link between both sides. Finally, our data imply that BMP4 activity is tightly regulated at transcriptional levels, by Activin and SHH, and post transcriptional levels, by BMP antagonists or the availability of other members of the BMP cascade.

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Gene expression evidence on the evolutionary origins of the vertebrate left/right pathway. Some unexpected experimental effects of TGFβ-related ligands in chick

Jonathan Cooke

There is substantial agreement on the functional pressures that would have underlain the development of highly co-ordinated and stereotypical, left-right asymmetrical packing of the extended tubelike viscera within the symmetrical and streamlined 'locomotory' vertebrate body. The symmetrical musculo-skeletal system in its various forms is an adaptation to an active, questing lifestyle that probably arose distinctively with vertebrate (as opposed to chordate) life. But because of the 'square-cube law', the enhanced metabolic and work-rate demands of that life require relative surface areas, particularly for gut and vascular systems, that ultimately dictate looping and/or coiling of those viscera for their functional adequacy. This is intuitively likely but has recently been nicely documented for the pumping ability per muscle mass of the amniote vertebrate heart. If many independently arising (in the embryological sense) organ systems are to develop this asymmetrical complexity and, at the same time, develop with reliably optimal function in different individuals, access to a central source of left/right asymmetry information by the various organ primordia is required. Their individual mechanisms triggering asymmetrical development can then each respond co-ordinately. Without such a mechanism, a major proportion of individual embryos would develop with conditions equivalent to those known as 'heterotaxias' (randomly disposed asymmetries) when they occur within contemporary vertebrate bodies. These are mildly to severely pathological, because the various internal symmetries have now co-evolved to aid functional co-operation between organ systems, as well as optimising their individual functioning and their spatial packaging. On comparative anatomical grounds, this inter-organ co-ordination system is monophyletic; it arose, or at least evolved to stability, once only, and so is preserved with the various organ systems reading it 'the same way round' in all vertebrate groups.

Assumptions within the field as to the evolutionary mode of origin of this central embryonic left-right system seem to vary much more. One assumption might be that, as part of the evolutionary transition basal to craniate chordates, or at least to all known extant and fossil vertebrates, a mechanism arose de novo for converting subcellular molecular chirality (co-ordinated among many cells once the major embryonic axes had been defined) into the asymmetrical expression of one or more gene products about the primitive midline. This could act as the foundation of the left, and right, intercellular signalling and transcription factor cascades whose further unravelling will doubtless inform much of the meeting. An alternative assumption would be that it is the bilaterally symmetrical locomotory body - or at least a return to it, after a much more distant truly bilaterian ancestry - which was the evolutionary novelty within a lineage leading to vertebrates, from a fundamentally non-symmetrical body plan in the immediate ancestor. The genetic foundations of this non-symmetry could have been retained, and co-opted to co-ordinate the development of the increasingly complex viscera within the vertebrate streamlined outer body, probably as it enlarged and became more active.

All that we currently know, about the mechanisms of arriving at co-ordinated visceral asymmetry within embryos of current vertebrates, is consistent with either of the above

opposed evolutionary scenarios. But Amphioxus and its development, and the motile larvae of various tunicate chordates, may represent outgroups that offer clues to the immediately pre-vertebrate condition as regards the genetic foundations of asymmetry. I argue that the developmental expressions in these forms of various genes, homologous with members of the vertebrate 'left-right' gene cascades, are evidence for the second scenario. This is because asymmetrical expressions of such genes are present, but without those evolved visceral asymmetries (in heart and gut) whose presence might have been thought to have 'co-opted' the genes and thus explained the expressions if vertebrates alone were considered. The Amphioxus ventral head, on the other hand, shows developmental evidence of profound asymmetry in the ancestry of this form.

Studies in *Xenopus* and, more recently, mouse have suggested that a TGFβ-related gene, Vg1 or Gdf-1, may play a special role near the initiation point of vertebrate asymmetrical gene expressions. In particular, it has been referred to as a left-right organiser or 'co-ordinator' in that its experimental mis-expression on the right in *Xenopus* can systematically reverse the co-ordinated development of asymmetrical anatomy, whereas for most 'left-right' genes, experimental duplication of expression on their 'wrong' sides results in randomisation of such co-ordinations, as might be expected from the removal of a normal differential cue. The putative time of action of the gene product (from *Xenopus* work) is early in gastrulation, but has been hard to further define. Additionally, there has been the possibility that overexpression experiments, and even experiments with injected dominant negative contructs for pathway components, may confuse effects distinctive to this gene's encoded ligand with those that might result from interference with signaling via the related nodal protein, that is also basally involved in left specification.

The chick Vg1/Gdf-1 homologue, cVg, is also expressed across relevant developmental periods, and its misexpressed product, like that of Vg1, causes primary axial duplications in both chick or *Xenopus*. We have recently compared the results, using transfected COS-cell grafting, of ectopically expressing proteins of either cVg, or the mouse nodal gene and its homologue cNR-1, to right and left of the midline at two stages in chick gastrulation. Results differ strikingly for the two genes, in a way that reinforces the idea that Vg has a unique role in left-right organisation, different from that of nodal. However, strong destabilisation of left-right development following LEFT over-expression of either gene, as well as right over-expression, was unexpected. This destabilisation was not accompanied by the same perturbations of normal downstream L-R gene expressions as were the expected ones that followed the right-sided grafts. The anatomy of 'reverse-looped' hearts following the right- and the left-overexpressions of both genes also appears different, but we have not yet been able to interpret this in anatomical detail. It appears that we have not yet finished understanding the differental roles of various TGFβ-related factors and their transduction components in the vertebrate left-right information cascade.

Modulation of the first steps in left-right axis formation

H. Joseph Yost

Heparan sulfate proteoglycans expressed on the *Xenopus* animal cap ectoderm have been implicated in transmitting left-right information to migrating heart and gut primordia during gastrulation. Syndecan 2 functions in the ectoderm to regulate heart and gut situs, upstream of known asymmetrically expressed genes. Syndecan 2 functions during early gastrulation and requires a functional link between the intracellular domain and multiple glycosaminoglycan attachment sites in the extracellular domain. Syndecan 2 binds Vg1, a TGFβ signaling molecule previously shown to coordinate left-right development. Directed expression of active Vg1 ligand restores normal situs in embryos with altered Syndecan 2. The results support a model wherein one of the earliest steps in left-right axis formation involves the modulation of Vg1 activity by Syndecan 2 on the surface of the embryonic ectoderm, and transmission of this left-right information to migrating mesoderm during gastrulation.

Session 3: Propagation of left-right signaling
Chair: Hiroshi Hamada

Genetic and biochemical analysis of nodal signaling in the mouse

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Loss of function analysis has shown that the TGF-β-like factor nodal is essential for mouse mesoderm development. In addition to an early role in gastrulation, nodal has been implicated in left-right development due to asymmetric expression at early somite stages and disruption of this pattern in mouse mutants with left-right patterning defects. However, definitive proof of nodal function in left-right patterning in the mouse embryo has been lacking because the null mutation blocks gastrulation. Therefore, we generated a nodal allele flanked by loxP sites (floxed) to permit conditional mutagenesis in conjunction with transgenic mice expressing Cre recombinase at appropriate stages and tissues. Unexpectedly, but fortuitously, we discovered that the floxed nodal allele is hypomorphic in the absence of any Cre mediated recombination. Embryos carrying the floxed and a nodal null allele undergo gastrulation but then display abnormalities at later stages that fall into three distinct phenotypic classes. Comprehensive analysis of the phenotype of one mutant class has provided conclusive evidence of the essential role nodal signaling plays in the proper left/right asymmetric development of the heart, lungs, vasculature and stomach (1).

Our analysis of the nodal hypomorph has revealed that Pitx2 and lefty-1 and -2 are downstream targets of nodal signaling in the process of left-right development. To define additional targets of nodal signaling we have utilized P19 pluripotent embryonal carcinoma cells, which provide a cell culture model system for differentiation events occurring during early mouse development. We have shown that P19 cells are responsive to recombinant nodal protein, and exploited this feature to dissect the intracellular components of the nodal signaling pathway (2). We are now exploiting the P19 system further to determine how the gene expression profiles of early embryonic cells change in response to nodal. We have compared P19 cells that overexpress nodal with normal P19 cells in a screen of micro-arrays of 15,000 genes expressed in the developing mouse embryo. This study has revealed a number of genes whose expression differs significantly in these two cell populations. Validation of these candidate nodal target genes is being carried out by analyzing expression in P19 cells treated with nodal protein. Further validation will involve analysis of candidate target gene expression in the developing wild-type and nodal mutant embryo.

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The role of nodal/lefty regulatory loops in left-right patterning

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Many lines of evidence suggest that *nodal* and *lefty* form positive/negative regulatory loops wherein Nodal induces expression of *nodal* and *lefty* and Lefty terminates this induction by inhibiting Nodal. This feedback mechanism can restrict the range and duration of Nodal signaling, and appears to play an essential role in various patterning events such as anterio-posterior patterning, mesoderm formation and left-right patterning.

We have recently investigated the role of Lefty2 in left-right patterning by analysis of mutant mice that lack asymmetric expression of *lefty2*. These animals exhibited various situs defects including left isomerism. As expected, the asymmetric expression of *nodal* was prolonged and the expression of *Pitx2* was up-regulated in the mutant embryos. The absence of Lefty2 conferred on Nodal the ability to diffuse over a long distance. Thus, Nodal-responsive genes, including *Pitx2*, that are normally expressed on the left side were expressed bilaterally in the mutant embryos, even though *nodal* expression was confined to the left side. Both GFP-Lefty2 and GFP-Nodal proteins could travel over a long distance in chick embryos, but the former could diffuse faster than the latter. These results suggest that Nodal is a long-range signaling molecule but that its range of action is normally limited by Lefty2, a feedback inhibitor that diffuses more efficiently than does Nodal. These data also support an idea that Nodal and Lefty form Turing's "reaction-diffusion system".

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Control of left-right axis specification by EGF-CFC genes

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Our studies in zebrafish have revealed that the interplay of extracellular factors of the Nodal (*cyclops* and *squint*), EGF-CFC (*one-eyed pinhead; oep*) and Lefty (*antivin*) families controls gastrulation in vertebrates: Nodal signals instruct cells to form mesoderm and endoderm; Oep acts as an essential cofactor in this process; and Lefty attenuates germ layer formation by blocking Nodal signaling. *Oep* is not only expressed during early development, but also in the midline and lateral plate at later stages, overlapping with the expression of *Nodal* and *Lefty* genes. To determine the role of EGF-CFC-mediated signaling during left-right development, we have rescued the early requirement for *oep* in germ-layer formation by injecting *oep* mRNA into maternal-zygotic *oep* mutants. These rescued embryos are viable but develop heterotaxia, and the expression of left-side specific genes is abolished. The direction of heart looping and the location of the pancreas are randomized. Moreover, in collaboration with M. Concha and S. Wilson, we have found that the location of the parapineal organ in the dorsal diencephalon is randomized. These results indicate that EGF-CFC signaling is not required for left-right asymmetry per se, but is essential to specify the laterality of the asymmetry. Similarly, our collaborators M. Shen and M. Muenke have found that mutations in mammalian *oep* homologues (mouse *Cryptic*; human *CFC1*) are associated with laterality defects in mice and humans. I will present experiments designed to determine how and where Oep-mediated signaling acts during left-right development.

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Left-Right axis development in *Xenopus laevis*

Christopher V. E. Wright

Of six *nodal-related* genes in *Xenopus*, only *Xnr1* is expressed asymmetrically in the left side of the tailbud stage embryo. It is expressed in a transient and dynamic fashion in the lateral plate mesoderm, in what appears to be a sweeping wave from posterior to anterior regions. *Xatv*, the *Xenopus lefty* ortholog, is also expressed in the left lateral plate mesoderm, with a timing slightly delayed compared to *Xnr1*. Our experimental results indicate that Xatv functions as a feedback inhibitor of Xnr1 signaling, and that it can function to inhibit the asymmetric expression of *Xnr1*. An Xnr autoregulatory enhancer located in an intron seems to be one of the critical determinants that determine the *Xnr1* left-sided expression pattern. We are currently studying how the asymmetric *Xnr1* expression is activated, the nature of the signals and tissues that cause the posterior-to-anterior shift in *Xnr1* expression, and if Xatv functions as a feedback inhibitor of Xnr signaling to ensure the transient expression of this Nodal signal to the embryo's left side. We are also beginning to explore the range of Xnr1 signaling in tailbud stage tissues, particularly in the sheet of lateral plate mesoderm, and the effect that negative feedback from Xatv regulates this property.

Arkadia is essential for specification of antero-posterior and left-right axes

P. Timmons, R. Andrew, V. Episkopou

Arkadia (Akd) mRNA is expressed in embryonic and extraembryonic lineages, and the protein is located in the nucleus. Akd null mutants establish anterior visceral endoderm but fail to develop anterior definitive endoderm (ADE), a node, or node-derived tissues, and therefore fail to maintain the most anterior embryonic structures1. Analysis of chimeras shows that Akd functions within the extraembryonic lineages to induce cells in the anterior primitive streak that express HNF3b, and are the precursors of the ADE and the node. Genetic experiments in mice, and frog embryo assays2, show that (1) Akd function is conserved, (2) Nodal is the signal that mediates Akd's function in node/organiser induction, and (3) Akd enhances Nodal signalling. Akd null embryos fail to turn and their heart tube does not loop, showing that Akd is also essential for left-right (L-R) axis specification. Chimeras generated with tetraploid WT extraembryonic lineages and Akd null ES cells develop a node and a head but still do not turn. This suggests that Akd's role in L-R axis formation is within the embryo and distinct from its role in node induction. Analysis of markers, including Nodal, indicates that gene expression that is normally restricted to the left side of the lateral mesoderm (LM) is missing in Akd null embryos. We are currently extending our analysis of gene expression in Akd null embryos, and investigating whether Akd and Nodal interact in L-R axis specification.

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Session 4: Asymmetry of the brain and other organs
Chair: Chris McManus

Molecular and morphological asymmetry in the dorsal diencephalon

Marnie Halpern

It has long been known that the diencephalic region of lower vertebrates shows morphological differences on the left and right sides. My laboratory has been studying left/right (L/R) asymmetries in the zebrafish dorsal diencephalon and developing molecular tools to explore how they arise. During embryogenesis, components of the nodal signalling pathway are transiently expressed on the left side of the brain in a region corresponding to the presumptive epiphysis or pineal organ. Previously, we showed that perturbation of nodal signalling alters the positioning of the pineal stalk, which normally emerges from the roof of the diencephalon at a left to medial position. Zebrafish and other teleosts develop a parapineal, typically located just to the left of the pineal organ. Mutations that perturb L/R patterning of the viscera also affect the laterality of the parapineal. A third asymmetry we are studying is associated with the dorsal habenular nuclei, which show distinct gene expression patterns on the left and the right. This feature can also be altered in zebrafish mutants; however, "left" habenular identity always corresponds with the position of the parapineal. We are testing the hypothesis that the parapineal, which migrates relative to the pineal to reside deep within the left brain, influences habenular L/R identity. Whether molecular asymmetries of the diencephalon are conserved evolutionarily is another issue we are actively exploring.

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Right and left handed brains

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Although the most obvious asymmetry in the body of vertebrates is of the heart and viscera, humans also show another conspicuous asymmetry in the brain, an asymmetry. Ninety percent of people are right-handed, with language typically located in the left hemisphere and visuo-spatial and non-verbal processes in the right hemisphere. Right and left-handedness are under the control of a single gene, which also determines language lateralisation, and explains the otherwise complex inter-relation of language and handedness. Understanding cerebral lateralisation could be of great importance in medicine and psychology, since atypical lateralisation occurs in a host of conditions, including dyslexia, stuttering, autism, and schizophrenia.

Although it might be thought that cerebral lateralisation is secondary to visceral lateralisation that is not the case, there being solid evidence that the majority of humans with situs inversus are *right-handed* (i.e. they are not the mirror image of those with situs solitus). That means there has to be a second control process determining lateralisation which is independent of visceral asymmetry, although it might well have developed from it, and share some of the underlying processes.

In this paper I will overview some of the biology and neuropsychology of handedness and cerebral lateralisation, and I will ask how handedness and language dominance might be related in evolutionary terms to visceral asymmetry. I will also speculate about possible ways of finding the important gene responsible for variation in handedness and cerebral asymmetry.

Left-right asymmetry in the embryonic gut of *Drosophila*

Petros Ligoxygakis, Maura Strigini, Michalis Averof

Most animals exhibit stable left-right asymmetries in their body. While significant progress has been made in elucidating the mechanisms that set up these asymmetries in vertebrates, very few studies on the specification of left-right asymmetry have been carried out in *Drosophila*, an organism that usually offers great opportunities for genetic and developmental research. The lack of such studies is often attributed to the idea that stable left-right asymmetries may not be subject to genetic control in *Drosophila*, as no reversals of stable left-right asymmetries have so far been observed in this species. We have focussed on the asymmetry of the proventriculus in the embryonic gut of *Drosophila*, a left-right asymmetry that is extremely stable in wild-type flies. We show that this asymmetry can be reversed by mutations in the dicephalic and wunen genes, that also cause reversals in the antero-posterior axis of the embryo relative to its mother. We consider two alternative possibilities for the initial 'breaking of symmetry' and suggest that the asymmetry of the proventriculus is initiated by a signal that is provided asymmetrically from maternal tissues.

The role of Nodal signalling in regulating the laterality of asymmetry in the epithalamus of zebrafish

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The epithalamus is a major subdivision of the diencephalon constituted by the habenular nuclei and pineal complex. Structural asymmetries in this region are widespread amongst vertebrates and involve differences in size, neuronal organisation, neurochemistry and connectivity. In species that possess a photoreceptive parapineal organ, this structure projects asymmetrically to the left habenula, and in teleosts it is also situated on the left side of the brain (reviewed in Concha and Wilson, 2001). In at least some extant vertebrate species, epithalamic asymmetries are established early in development suggesting a genetic regulation of asymmetry. Although the genetic mechanisms by which neuroanatomical asymmetries are established remain obscure, we have started to address the mechanisms underlying laterality decisions by using the zebrafish as a model system. Brain asymmetry in the wild-type zebrafish embryo is characterised at early stages by asymmetric expression of Nodal pathway genes on the left side of the epithalamus, e.g. the Nodal ligand cyclops (cyc) and the downstream effector of Nodal signalling pitx2 (see list of references in Concha et al. 2000). At later stages, neuroanatomical asymmetries consist of a parapineal organ located on the left side and the habenula enlarged on the left side. Analysis of zebrafish embryos with compromised Nodal signalling and with midline defects reveals that cyc and pitx2 are either bilaterally symmetric or absent in the epithalamus. Importantly, neuroanatomical asymmetries still develop in both situations although the laterality is disrupted resulting in heterotaxic randomisation. These results indicate that Nodal signalling is not required for the development of asymmetry per se but is required to determine the laterality of asymmetry by biasing an otherwise stochastic laterality decision to the left side of the epithalamus (Concha et al. 2000).

We are currently assessing the roles of other signalling pathways in the establishment of neuroanatomical asymmetries. Furthermore, in collaboration with Richard Andrew and his colleagues, we hope to determine if alterations in neuroanatomical laterality are correlated with alterations in the laterality of behavioural asymmetries.

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Phylogenetic patterns of asymmetry variation in animals and their evolutionary significance

A. Richard Palmer

Because conspicuous, external, morphological asymmetry has evolved independently in many animal groups, the frequency of alternative evolutionary pathways to fixed asymmetry may be assessed via phylogenetic reconstruction. Using three morphological asymmetry states that may be compared across widely differing taxa (a- symmetry, b- antisymmetry= right or left at random within a species, and c- directional asymmetry= always right or always left within a species) and two stages at which asymmetry appears during development (early vs late), I tallied 140 inferred evolutionary transitions between asymmetry states from 11 classes in 5 animal phyla. These tallies revealed that 1) directional asymmetries (state-c) arose directly from symmetrical ancestors (state-a) proportionally more frequently when asymmetries were early-developing, 2) antisymmetry (state-b), either as an end state or as a transitional stage between symmetry and directional asymmetry, was confined almost exclusively to late-developing asymmetries, and 3) directional asymmetries (state-c) arose almost as frequently from antisymmetrical ancestors (state-b) as from symmetrical ones (state-a). Because antisymmetry typically signals that symmetry is broken by external environmental stimuli or epigenetic effects during development, the unexpectedly high proportion of evolutionary transitions from antisymmetric ancestors to directionally asymmetric descendents suggests that environmental effects and genetic assimilation may have played a more significant role than generally acknowledged in the evolution of directional asymmetries.

Session 5: Towards left-right specific morphogenesis
Chair: Juan Carlos Izpisúa Belmonte

The rabbit, a second mammalian model organism to study of early laterality decisions

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The genetic cascade of events resulting in asymmetric morphogenesis and placement of the various organs in the thorax and abdomen has best been worked out in the chick (1). Following the intitial breakage of bilateral symmetry in the organizer at st. 4/5 the secreted growth factors *shh* and *FGF8* were implicated in the transfer of the original asymmetric signal from the midline to the periphery. As a result the TGFß-like growth factor *nodal* and the transcription factor *Pitx2* become asymmterically activated in the left lateral plate mesoderm. Whereas the activity of *nodal* is very transient, *Pitx2* stays on during asymmetric organ morphogenesis.

While the *nodal-Pitx2* pathway seems to be conserved between the different classes of the vertebrates, differences exist with respect to the roles of *shh* and *FGF8*. *shh* in the chick displays left-asymmetric activity in the node and is required for left-sided activation of *nodal* (2). In the mouse *shh* functions in the midline or on the right side (3). *FGF8* plays a right-sided role in chick (4), but it is a left determinant in mouse (3). We use the rabbit as a second mammalian model system to study evolutionary aspects of early axis formation. Rabbit embryos, like chick and human, develop via a flat blastodisc, unlike mouse embryos which are cup-shaped at that stage. Due to late implantation and larger size rabbit embryos at the gastrula/neurula stage can be much easier isolated, manipulated and cultured *in vitro* compared to mouse embryos of similar developmental age.

We have cloned marker genes and established a culture system to study early laterality. *shh* and *FGF8*, like in mouse, are symmetrically expressed at the node, while *nodal* and *Pitx2*c, as in all vertebrates, show left-asymmetric activity. Right-sided misexpression of activin, a *nodal*-related growth factor, resulted in ectopic expression of *nodal* and *Pitx2*c, demonstrating that the rabbit embryo is competent to ectopically induce the *nodal* pathway on the right side of early neurula embryos, and that the *nodal-Pitx2*c pathway is conserved. *FGF8*, however, did not induce ectopic *nodal* on the right side, indicating differences to the mouse. Left-sided application of *FGF8*, however, repressed *nodal*, as has been reported for chick. A comparison of *FGF8* and *nodal* expression in early streak stage embryos reveal mutually exclusive patterns of mRNA localization. Because *FGF8* is not asymmetrically expressed in the rabbit, our data suggest a role for *FGF8* in localizing *nodal* mRNA to both sides of the notochord at early somite stages.

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Induction and early patterning of the heart

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Two Wnt antagonists, Dickkopf-1 and crescent, are potent inducers of cardiac tissues from normally non-cardiogenic *Xenopus* or chick mesoderm. Inhibition of b-catenin signaling also initiates cardiogenesis, indicating that the canonical Wnt pathway must be blocked. Conversely, overexpression of Wnts that are capable of activating the canonical b-catenin pathway inhibit native heart formation. Since expression of Dkk1 and Crescent is confined to the Spemann's organizer region in *Xenopus*, these proteins are presumed to act either upstream of, or in parallel with, an additional endodermal factor that has been shown to be necessary by classical graft studies. Although Crescent encodes a secreted frizzled-domain Wnt receptor, the basis for Dkk1 function is not clear. Domain analysis, however, demonstrates that both heart-inducing and Wnt antagonist potency reside within the carboxyl terminal cysteine rich region. Artificially induced heart tubes beat rhythmically and loop. Importantly, however, unlike native heart tubes that develop in organ culture, the artificially induced tubes lack distinct atrial and ventricular character as well as expression of Pitx2, which is normally left-sided. This result indicates that looping morphogenesis does not proceed by normal means and that the artificial hearts might be useful to explore Pitx2-induced morphogenetic processes.

Role of Bmps in the specification of the left-right axis in the chick embryo

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In vertebrates the specification of the left-right axis occurs during gastrulation integrated with the elaboration of the basic body plan (Capdevilla et al., 2000). The breaking of the initial embryo symmetry appears to be a complex task for which different species have developed different strategies. Subsequently vertebrate embryos converge into a "left-right phylotypic stage" characterized by the expression of *Nodal* and *Pitx2* genes on the left lateral plate mesoderm (Yost, 2001).

In chick (probably all avian embryos; Levin, 1998), the early phase of left-right specification involves the establishment of small asymmetric domains of expression of signaling factors on the node (Levin, 1995). Interactions between these signaling molecules result in the late broad asymmetric expression of *Nodal* and its target *Pitx2* gene in the left lateral plate mesoderm.

Several observations implicate members of the TGFβ superfamily in the early and late phases of left-right specification. In the chick, activin activity on the right side of the node is initially crucial to adequately limit *Shh* and *fgf8* expressions. Also an asymmetric right expression of *Bmp4* in the node has been described in chick embryos (Monsoro-Burq and Le Douarin, 2000). Later activation of *Nodal* expression depends on the precise antagonism of Bmp signaling by *Caronte* (Rodriguez-Esteban, et al. 2000; Yokouchi et al., 2000; Zhu et al., 1999). In other vertebrates (zebrafish, *Xenopus* and mouse), there is also abundant evidence indicating the implication of Bmps in the control of laterality. For example, interference with Bmp signaling, either at the extracellular or intracellular level, results in alterations of left-right development (Yost et al., 2001).

We have further analyzed the function of Bmp signaling during the initial and late phases of left-right asymmetry specification in the chick. We will describe and discuss our results in the context of the current models for laterality specification.

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Regulation of left-right asymmetry by thresholds of pitx2c activity

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Although much progress has been made in understanding the molecular mechanisms regulating left-right asymmetry, the final events of asymmetric organ morphogenesis remain poorly understood. The phenotypes of human heterotaxia syndromes, in which organ morphogenesis is uncoupled, have suggested that the early and late events of left-right asymmetry are separable. The pitx2 homeobox gene plays an important role in the final stages of asymmetry. Here, we used two new pitx2 alleles that encode progressively higher levels of pitx2c in the absence of pitx2a and b, to show that different organs have distinct requirements for pitx2c dosage. The cardiac atria required low pitx2c levels, while duodenum and lungs utilized higher pitx2c doses for normal development. As pitx2c levels were elevated, the duodenum progressed from arrested rotation to randomization, reversal, and finally normal morphogenesis. In addition, abnormal duodenal morphogenesis was correlated with bilateral expression of pitx2c. These data reveal an organ-intrinsic mechanism, dependent upon dosage of pitx2c, governing asymmetric organ morphogenesis and provide insight into the molecular events that lead to the discordant organ morphogenesis of heterotaxia.

Imposition of asymmetry on a symmetric DNA substrate by a recombinase enzyme

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Considerations of biological asymmetry at the level of the organism have been largely rooted in genetics and driven by notions of gene regulation. Biological systems also abound in asymmetry at the molecular level. Most protein enzymes are chiral reagents, and treat symmetric centers in molecules as asymmetric. The asymmetry we are interested in concerns a DNA recombination system: very much smaller than the organism, but considerably larger than small metabolites such as sugars or amino acids.

The DNA recombinase Flp acts on a pair of target sites, each 34 bp long. There is central 8bp asymmetric sequence within this site (called the spacer), that is flanked by two essentially identical 13 bp elements in head to head orientation. Each of these flanking sequences binds a monomer of Flp. Two recombination partners bound by four Flp monomers come together in a 'synapse' of fixed geometry, and carry out strand cutting and joining.

Because of the asymmetry of the core sequence, the recombination sites have a left to right orientation, which fixes the direction of 'cutting and pasting' during recombination. As a result, when two head to head sites recombine, they invert the DNA segment between them. When two head to tail sites recombine, they excise the intervening DNA. When, the sites are symmetrized (abolishing left-right asymmetry), inversion or excision occurs with equal probability.

First, we use a topological analysis to show that the asymmetric sites align in the antiparallel mode within a functional recombination synapse. We then extend this analysis with symmetrized substrates to show that the recombinase desymmetrizes the DNA within the synapse. The breaking of symmetry fixes a single orientation for exchanging DNA strands, regardless of the inherent symmetry or asymmetry of the recombination sites.

POSTERS

A molecular basis of heterotaxy caused by Zic3 dysfunction

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I will show that a zinc finger protein, Zic3, mediates the left-sided signal from the initial activin-like signal to determinative factors such as pitx2 in *Xenopus* embryos. Overexpression of Zic3 on the right side of the embryo altered the orientation of heart and gut looping, concomitant with disturbed laterality of expression of Xnr1 and pitx2, both of which are normally expressed in the left lateral plate mesoderm. The results indicate that Zic3 participates in the left-sided signal upstream of Xnr1 and pitx2. At early gastrula, Zic3 was expressed not only in presumptive neuroectoderm but also in mesoderm. Correspondingly, overexpression of Zic3 was effective in the L-R specification at the early gastrula stage as revealed by a hormone-inducible Zic3 construct. The Zic3 expression in the mesoderm is induced by activinbB or BVg1, which are also involved in the left-sided signal in L-R specification. Therefore, an activin-like signal is a potent upstream factor of Zic3 to establish the L-R axis. These results provide a molecular basis for human heterotaxy, which is an L-R pattern anomaly caused by a human ZIC3 mutation. In addition, recent findings concerning the function of the Zic family proteins would be presented.

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Differential wing asymmetry related to migratory habits: an interpopulation study

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Asymmetry occurs when small, random deviations from perfect bilateral symmetry arises for a morphological trait and occurs when an individual is unable to cope with environmental and genetic stresses during the development of the trait. The effects of asymmetry on performance has been several times stated, including relationships between asymmetry degree and aerodynamics. Both wing and tail asymmetry increase the power require for flight at slow speeds by affecting the distribution of the lift across the aerodynamic surface which introduces large rolling and yawing forces and decrease the lift generated. This should affect efficiency to refuel at stopover sites increasing migration time and therefore delaying arrival time at breeding sites. Avian aerodynamics provides an example of the influence of asymmetry on locomotion, and then migration should be affected by asymmetry. Birds and many other animals are migratory spending part of the year outside the breeding grounds. Long-distance migration requires much effort. The relationship between morphology and migration has been studied interspecifically and intraspecifically. Biomechanics of migration have been reviewed recently. However, there is no studies on the relationship between different migratory habits including migration distance among different populations of the same species and asymmetry of morphological traits related to flight. Emberiza schoeniclus is a bird species that shows different populations with different migratory habits including sedentary, medium and long distance migration. Therefore it represents a good model to test predictions of differential asymmetry related to differences in migratory behaviour. The aim of this paper is to test whether some differences in asymmetry in a highly functional trait such as wing length arise among populations in relation to the functional importance of a behavioural trait such as migration. Specifically we predict 1) that if wing length is related to migration distance, long distance populations should have longer wings than medium and sedentary populations, therefore we expect the follow ordination from longer to shorter wings (Swedish > German > Spanish population). And 2) asymmetry should be higher in sedentary populations than in migratory populations decreasing in relation to migration distance as the absence of a very high stress pressure such as migration allows a release of the canalization processes during the development. Therefore, asymmetry should be ordered as follows from high to low asymmetry (Spanish > German > Swedish populations). Results showed that there were significant differences in wing length among populations and that such differences were ordered in relation to migration distance as expected being larger in the swedish population, the long distant. Wing asymmetry, both absolute and relative, showed differences among populations in relation to migration distance as expected.

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Pitx and left-right axis evolution in chordates

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Anterior-Posterior Patterning and Dorsal-Ventral patterning are well studied in a diverse range of animals across the animal kingdom. Conserved genetic pathways have been discovered and discussed from an evolutionary standpoint. Left-Right asymmetry or the specification of the left-right axis, though well studied in vertebrates, remains enigmatic and poorly understood outside of this evolutionary lineage (Capdevila, J. et al 2000). During the last few years research in left-right asymmetry of vertebrates has highlighted the conservation of some genes important to the left-right axis. Two genes in particular have been found to be involved in the left-right axis in all species of vertebrates studied so far. Nodal and pitx2c are expressed in the left lateral plate mesoderm after the A-P and D-V axes have been set up and are important for specifying laterality (Ryan et al 1998). Ptx in flies has no reported role in laterality and no asymmetric expression during development (Vorbruggen, 1997), while nodal has no obvious fly homolog. In our lab we have isolated ptx genes from Lamprey, Ciona and Amphioxus and their expression patterns and role in left-right asymmetry will be discussed focusing on the comparative developmental biological and evolutionary aspects of the available data.

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The regulation of nodal signaling in left-right axis determination

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Vertebrates have internal asymmetries along the left-right (L-R) axis revealed by asymmetric organ arrangement about the midline. In all vertebrates examined, members of the Nodal signaling pathway are expressed asymmetrically on the left in the lateral plate mesoderm (LPM). In zebrafish larvae there is additional asymmetric expression of these components on the left in the diencephalon. Asymmetric gene expression, coupled with ectopic expression studies, has suggested that Nodal acts as a global signal for "leftness" in the developing embryo, but an essential role for Nodal signaling had not been shown. The zebrafish gene one-eyed-pinhead (oep) is a member of the EGF-CFC family of proteins that are required cofactors for Nodal signaling. Zebrafish mutant for both maternal and zygotic oep (MZoep) lack all mesoderm and endoderm and thus the effect on the L-R axis cannot be assessed in these embryos. To overcome this limitation, we rescue MZoep mutants by RNA injection at the one-cell stage. This allows for the formation of mesoderm and endoderm and the resulting larvae appear wildtype. However, these larvae have defects in organ placement along the L-R axis in both the viscera (heart/pancreas/ liver) and within the brain (parapineal/habenula). We thus refer to these larvae as late zygotic or LZoep mutants. L-R defects can be observed earlier in LZoep embryos as asymmetric gene expression in the LPM and diencephalon is never established. Similar results were obtained in zebrafish schmalspur (sur)mutants, that have defects in the Fast1 transcription factor which transduces the Nodal signal, and in mouse knockouts for the EGF-CFC gene Cryptic. In all of these mutants, organs still obtain asymmetric positions, but these positions are randomized with respect to the midline. This indicates that Nodal signaling is not required for the generation of asymmetry per se, but is required to direct these asymmetries. Together these results provide the first evidence for a conserved requirement for Nodal tube. A later oep dependent step is required in order to overcome this repression and allow for gene expression to occur properly on the left. Transplant experiments and manipulations in LZoep embryos supports a similar model for Nodal signaling in the LPM. In addition to studies on Nodal signaling in L-R patterning, we are attempting to map and clone several genes that affect L-R development in the zebrafish. Our recent results on this work will also be presented.

The role of FGF8 in left-right axis specification in the rabbit

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FGF8 was implicated in the transfer of the original asymmetric signal from the midline to the periphery. However, its role in chick and mouse seems to differ. While FGF8 plays a right-sided role in chick, it is a left determinant in mouse. We use the rabbit as a second mammalian model system to study evolutionary aspects of early axis formation. Rabbit embryos, like chick and human, develop via a flat blastodisc, unlike mouse embryos which are cup-shaped at that stage. We have cloned marker genes and established a culture system to study early laterality decisions. shh and FGF8, like in mouse, are symmetrically expressed at the node, while nodal and Pitx2c, as in all vertebrates, show left-asymmetric activity. Right-sided misexpression of activin, a nodal-related growth factor, resulted in ectopic expression of nodal and Pitx2c, demonstrating that the rabbit embryo is competent to ectopically induce the nodal pathway on the right side at early neurula stages, and that the nodal-Pitx2c pathway is conserved. FGF8, however, did not induce ectopic nodal on the right side, indicating differences to the mouse. At the early somite stage (0-2 somites) FGF8 did not affect the left-sided nodal expression. Further experiments addressing the functions of FGF8 are in progress and will be discussed.

Genetic manipulations affecting left-right asymmetry in zebrafish

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Owing to the transparent embryos, powerful genetics and rapid development, zebrafish are ideal for investigating organogenesis and embryonic patterning. We are conducting a systematic mutational analysis of zebrafish embryos to identify new regulators of left-right asymmetry. It has been shown in several species that the left-expression of *nodal* in the LPM correlates to normal development of the LR axis, since *nodal* controls a number of target genes (such as *pitx*2) required for asymmetric organogenesis. Results from our lab demonstrate an instructive role for *Pitx*2 in directing asymmetric development of the heart and gut. Our focused screen will utilize a transgenic zebrafish stain in which eGFP is driven by a *pitx*2 enhancer, resulting in asymmetrically expression in the left LPM. Upon injecting a 10kb promoter fragment of *zPitx2* fused to eGFP, we have generated some high percentage mosaics that will be outbred to establish stable lines. We will mutagenize wild-type adult males with ENU and mate them to homozygous females derived from dominant eGFP insertion lines. Haploids produced from F1 female progeny will be examined live (at 20-22 somites) under fluorescent stereomicroscopy for altered asymmetric eGFP expression. Such a screen on living embryos viewable over time will facilitate isolation of mutations that affect asymmetric expression.

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Chicken *CFC* controls *LEFTY1* expression in the embryonic midline and *NODAL* expression in the lateral plate

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Proteins of the EGF-CFC (Cripto (Cfc2), FRL-1, Cryptic (Cfc1)) family are characterized by a modified EGF domain and a cysteine-rich CFC region, a N-terminal signal peptide and a C-terminal GPI anchorage site. Genetic data provided evidence for a role of members of the EGF-CFC family in Nodal signalling and thereby during the establishment of left-right asymmetry in zebrafish and mouse. With the purpose to further elucidate these processes in the chick embryo, we isolated a chicken member of the family from a HH stage 3-6 chicken library. Being the only member isolated in chick so far we named it chicken CFC. Expression was found prior to gastrulation in the epiblast layer and during gastrulation in the primitive streak, the forming notochord, Hensen's node and the lateral plate mesoderm (LPM). Like a number of other genes in chick implicated in left-right axis formation, chicken CFC was expressed asymmetrically around Hensen's node with expression being consistently stronger on the left side. Later, expression was found within the heart anlage, in the posterior part of the primitive streak and surrounding mesendoderm. After tubular heart formation the myocardial layer showed expression of CFC. Implantation of BMP and Noggin secreting cells adjacent to the LPM established that CFC expression here depended on BMP signaling, while midline expression was independent of this pathway. In the paraxial mesendoderm the implantation of beads carrying Activin also induces CFC expression strongly, while the extracellular domain of ActRIIA, a dominant negative form the receptor, abolishes expression of CFC when implanted in the notochord at HH stage 5. Therefore, an Activin-like signal might control the midline expression of chicken CFC. Overexpression of different EGF-CFC peptides on the right side of Hensen's node randomized heart looping without affecting the expression of laterality markers such as SHH, CARONTE, NODAL, cSnR, PITX2 and NKX3.2. Application of antisense oligonucleotides (ODN) targeted against two separate regions of chicken CFC mRNA to the node region of HH stage 5 embryos in New culture also resulted in randomization of heart situs compared to control embryos. However, bilateral expression of NODAL, CARONTE, PITX2 and NKX3.2 and loss of LEFTY1 expression in the midline was observed. If, on the other hand, ODNs were applied to the lateral plate, the left sided NODAL expression was abolished. The data presented are in accordance with data from the null mutation of mouse Cfc1 where the expression of nodal, lefty1 and Pitx2 is absent and indicate that chicken CFC has two distinct functions 1) in the LPM acting as a putative cofactor of Nodal signaling and 2) in the establishment of the midline barrier via control of LEFTY1 expression.

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